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Epidemiology of human coronavirus NL63 infection among hospitalized patients with pneumonia in Taiwan

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Short title: **HCoV-NL63 infection in Taiwan**

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Abstract

Background: Human coronavirus (HCoV) NL63 is recognized in association with upper or lower respiratory tract illnesses in children. This study surveyed the prevalence of HCoV-NL63 and influenza viruses in patients with influenza-like illness in Taiwan during 2010-2011.

Methods: Throat samples from 107 hospitalized patients with pneumonia and 175 outpatients with influenza like illness were examined using real time PCR assays with virus-specific primers, and then virus-positive specimens were further confirmed by sequencing the PCR products.

Results: HCoV-NL63 infection was identified in 8.4 % (9/107) of hospitalized patients with pneumonia, but not found in outpatients with influenza like illness. Age distribution of HCoV-NL63 infection in hospitalized patients with pneumonia indicated the group aged 16-25 years (20%) as the highest positive rate than other groups, exhibiting a similar age-specific pattern to influenza A/H1N1infection, but not influenza A/H3N2 and B infections in hospitalized patients. Prevalence seasonality of HCoV-NL63 infection was late winter, overlapping the highest peak of influenza A/H1N1 epidemic during the period December 2010 to March 2011 in Taiwan. Co-infection of HCoV-NL63 and influenza A/H1N1 was detected in 3 hospitalized patients. Clinical manifestation analysis indicated that the main symptoms for HCoV-NL63 infection included fever (88.9%), cough (77.8%), and

pneumonia (100%). Co-infection caused significantly higher rates of breathing difficulties, cough and sore throat than those of single infection with HCoV-NL63 and influenza A/H1N1. Phylogenetic analysis indicated a low level of heterogeneity between Taiwan and global HCoV-NL63 strains.

Conclusion: Understanding epidemiology of HCoV-NL63 in Taiwan provides an insight for worldwide surveillance of HCoV-NL63 infection.

Keywords: Human coronavirus NL63, age distribution, seasonality, phylogenetic analysis, pneumonia

Introduction

Human coronavirus (HCoV) NL63 is identified from the nasopharyngeal aspirate specimen of a 7-month-old child with coryza, conjunctivitis, fever and bronchiolitis in 2004, as the member of the Coronaviridae family, like other HCoVs HCoV-229E, HCoV-OC43, SARS-CoV, HCoV-HKU1, and MERS-CoV.[1, 2] CoV genome is a near 30 kb positive-strand RNA with a 5' cap and 3' poly (A) tract that contains 14 open reading frames (ORFs) encoding for non-structural proteins and structural proteins (conserved spike (S), envelope (E), membrane (M), and nucleocapsid).[1, 2] The 5' proximal and largest of these ORFs encodes two large overlapping polyproteins replicase 1a and 1ab (~ 450 kDa and ~750 kDa, respectively) processed to produce nonstructural proteins (nsps) primarily involved in RNA replication. Two specific embedded proteases, papain-like (PLpro) and 3C-like (3CLpro), mediate processing of 1a and 1ab precursors into 16 nsps (termed nsp1 through nsp16). Phylogenetic tree analysis of CoV genomes indicates HCoV-NL63 forming a subcluster with HCoV-229E, PEDV (porcine epidemic diarrhoea virus), and Bat-CoV, as assigned the alphacoronavirus coronaviruses.[3] Among all genes, HCoV-NL63 nucleocapsid (N) shows a low percentage of nucleotide and amino acid identity compared to other CoVs.[2]

HCoV-NL63 infection is usually surveyed in children with upper or lower

respiratory tract illnesses. HCoV-NL63 infection is found worldwide, but has rare positive rates by RT-PCR assays.[4-7] The positive rate of HCoV-NL63 infection in children ranges from 1.2% in Japan,[7] 1.3% in Taiwan,[8] 2.1% in Australia,[4] 2.3% in Belgium,[9] 2.5% in Canada,[10] to 7% in Swiss.[11] For adults, HCoV-NL63 infection is identified in 9.3% of respiratory tract illness patients under the age of 20 in France.[12] HCoV-NL63 infection is predominant in the winter season in Australia, Belgium, Canada, France, Germany and Japan,[4, 6, 9, 10, 12, 13] but spring and summer in Hong Kong,[5] as well as autumn and winter in Taiwan.[8] This study analyzes 2010-2011 surveillance data for HCoV-NL63 and influenza virus infection in hospitalized patients with pneumonia and outpatients with influenza-like illness in Taiwan, indicating the prevalence and phylogenetic analysis of HCoV-NL63 infection. Our results demonstrate a comprehensive comparison between HCoV-NL63 and influenza virus infection in hospitalized patients and outpatients.

Materials and Methods

Study Design

The study recruited 107 hospitalized patients with pneumonia and 175 outpatients with influenza-like symptoms in China Medical University Hospital (CMUH, Taichung, Taiwan) during 2010-2011. One throat swab was taken from each

indicated patient, and then examined using RT-PCR and real-time RT-PCR for detection of HCoV-NL63, influenza viruses A/H1N1, A/H3N2 and B. We followed guidelines established by the China Medical University Hospital Institutional Review Board.

RT-PCR, real-time RT PCR and sequencing

Human coronavirus NL63 provided by Dr. Lia van der Hoek (Academic Medical Center, The Netherlands) propagated in LLC-MK2 cells that grow in Modified Eagle's Medium supplemented with 2 mM L-Glutamine, 50 μg/ml penicillin, 50 μg/ml streptomycin, 100 μg/ml neomycin and 10% fetal bovine serum. A QIAamp Virus RNA Mini Kit (Qiagen) was used to extract viral RNA from clinical samples and supernatant of infected cells with HCoV-NL63, influenza A and B viruses as the positive controls. For detection of HCoV-NL63 infection, a two-step RT-PCR using SYBR Green I was used. The specific primer pair for HCoV-NL63 N gene (nucleatides 26416-26666) was forward primer 5'- CTGATGGTGTTGTTTGGGTT GC-3' and reverse primer 5'-AGAATCAGAACGAGTGCGAGAC-3'. Real-time PCR reaction mixture contained 2.5 μl of cDNA (reverse transcription mixture), 200 nM of each primer in SYBR Green I master mix (LightCycler TaqMAn Master, Roche Diagnostics). PCR was performed with amplification protocol consisting of 1

118 cycle at 50℃ for 2 min, 1 cycle at 95℃for 10 min, 45 cycles at 95℃ for 15 sec, and
119 60℃ for 1 min. Amplification and detection of specific products were conducted in
120 ABI PRISM 7700 sequence detection system (PE Applied Biosystems). For typing of
121 influenza A and B viruses as well as subtyping of H1 and H3, RT-PCR and real-time
122 RT-PCR assays were performed, as described in our prior report.[14]
123
124 **Phylogenetic analysis**
125 To confirm the real-time RT PCR assays, the products of nested RT-PCR for
126 HCoV-NL63 1a gene were further sequenced. The primers for nested RT-PCR were
127 5'-CTTTTGATAACGGTCACTATG-3' (SS 5852-5P) and
128 5'-CTCATTACATAAAACATCAAA CGG-3' (P4G1M-5-3P) in the first PCR; and
129 5'-GGTCACTATGTAGTTTATGATG-3' (P3E2-5P) and
130 5'-GGATTTTTCATAACCACTTAC-3' (SS 6375-3P; coordinate 6313) in the nested
131 PCR, described in a previous report.[15] Nucleotide sequences of 1a gene from the
132 product of nested RT-PCR were sequenced and used for phylogenetic tree analysis.
133 Reference sequences were chosen from GenBank (www.ncbi.nlm.nih.gov/genbank).
134 Genotypes and genotypic relationships for HCoV-NL63 1a genes were identified by
135 BioEdit 7.0.8 program (North Carolina State University at Raleigh,
136 http://www.mbio.ncsu. edu/BioEdit/bioedit.html) to align sequences with reference

sequences. Resulting datasets constructed phylogenetic trees for HCoV-NL63 1a genes, using MEGA v. 5.2 software (http://www.megasoftware.net/). After maximum-likelihood phylogenetic analyses in 1000 bootstrap replicates, branch bootstrap values above 60% or p-values of <0.05 clustering with specific genotype strains were determined. Cluster robustness could not all be statistically rated at 75% bootstrap due to huge size and highly genetically similarity of data sets, we used 60% to identify epidemic clusters. For further support of lower bootstrap values in cluster node, the ML tree confirms statistical significance ($p<0.05$) in each cluster node.

Statistical analysis

Statistical Package for the Social Sciences (SPSS) 12.0 software, two-tailed test, Chi-square test, and Fisher`s exact test were used to analyze all data. Statistical significance between both groups was noted at $p < 0.05$.

Results

Sensitivity of two-step real-time PCR with SYBR Green I

To examine the sensitivity and specificity of HCoV-NL63 detection, viral genomes were extracted from 200 μl of diluted supernatant containing 10 to 1000 pfu/ml of HCoV-NL63, and then quantitated using two-step real time PCR assays.

The C_t values were 30 for 2 copies, 27 for 20 copies, and 24 for 200 copies of HCoV-NL63, respectively. Melting curve analysis revealed HCoV-NL63 N-specific amplicon melting at 81 °C. The PCR products were separated using 2% agarose gel electrophoresis, where 251-bp band was clearly observed in the PCR reactions with 20 and 200 copies of HCoV-NL63 post gels stained with ethidium bromide. The results indicated that the detection limit of the two-step real time PCR assay was near 20 copies of HCoV-NL63. In addition, cultured supernatants of coxsackie virus 16, enterovirus 71, influenza viruses were not detectable using two-step real time PCR assay with HCoV N-specific primers. Therefore, the two-step real time PCR assay with HCoV N-specific primers had high sensitivity and specificity, as applicable for high throughput detection of HCoV-NL63 infection.

Surveillance of HCoV-NL63 infection in Taiwan.

A total of 282 throat swabs were taken from 107 hospitalized patients and 175 outpatients at the university hospital in central Taiwan from April 2010 to December 2011. All swabs were screened, using rapid diagnostic tests to detect HCoV-NL63, influenza A/H1N1, A/H3N2 and B viruses. In hospitalized patients, positive rates of real time PCR detection were 8.4 % (9/107) for HCoV-NL63, 15.9% (17/107) for influenza A/H1N1, 8.4 % (9/107) for influenza A/H3N2, and 4.7% (5/107) for influenza B, respectively (Table 1, Figure 1A). Importantly, co-infection of

HCoV-NL63 and influenza A/H1N1 was identified in 3 hospitalized patients. However, HCoV-NL63 infected was not found in outpatients with influenza-like illness (Table 2, Figure 1B). Lower positive rates of influenza A/H1N1 (13.1%), A/H3N2 (2.9%) and B (0.6%) were discovered in outpatients. Age distribution of HCoV-NL63 positive cases ranged from 3 to 79 years (Figure 1A). Interestingly, the prevalence of HCoV-NL63 was the second highest among adults aged 76-85 (1/7, 14.7%), and the highest among young people aged 16-25 (1/5, 20.0%) (Figure 1A). In contrast to HCoV-NL63, the positive rates in hospitalized patients were the highest among adults aged 66-75 (3/10, 30.0%), and the lowest among groups aged < 5 (0/16, 0%) for influenza A/H3N2 infection, as well as the highest among adults aged 36-45 (4/14, 28.6%), second highest among adults aged 56-65 (3/13, 23.1%), and lowest among the group aged 16-25 (0/14, 0%) for influenza A/H1N1 infection (Figure 1C). In addition, positive rates of influenza A/H1N1 in outpatients ranked the highest among adults aged 26-35 (1/5, 20.0%), second highest among children aged 2-5 (17/92, 18.5%), and lowest among the group aged >36 (0/18, 0%) (Figure 1D). Results indicated age-specific distribution of HCoV-NL63, influenza AH1N1, A/H3N2 and B-positive cases had different patterns.

Seasonality of HCoV-NL63 infection in Taiwan.

HCoV-NL63 infection was identified between January and February 2011, as late winter in Taiwan (Figure 2A). In contrast to HCoV-NL63, influenza A/H3N2 infection was predominant between August and December 2010, whereas positive rate of influenza A/H1N1 proved second highest in November 2010 and highest in January 2011, as well as declined after February 2011 (Figures 2B and 2C). No significant difference between hospitalized patients and outpatients was observed in the seasonality of influenza A/H1N1 and A/H3N2. The results revealed that seasonality of HCoV-NL63 infection overlapped the periods of influenza A/H1N1 circulation in Taiwan. Importantly, co-infection of HCoV-NL63 and influenza A/H1N1 appeared in 3 hospitalized patients over 40 years of age in February 2011.

Clinical association of HCoV-NL63 infection

Patients in the study were divided into 6 groups including negative, HCoV-NL63 positive, influenza A/H1N1 positive, A/H3N2 positive, B positive and co-infection. The clinical data of each group among hospitalized patient and outpatients were shown in Tables 1 and 2. Of hospitalized patients, the average age (41.7 years) of HCoV-NL63 positive group was higher than negative, but lowers than influenza A/H1N1 positive, and B positive groups. Clinical symptoms of HCoV-NL63 positive group had breathing difficulties (55.6%), cough (77.8%), fever (88.9%), pneumonia

(100%), myalgia (44.44%), and sore throat (11.8%), as similar to those of influenza A/H1N1 infection. However, co-infected patients presented significantly higher incidences in breathing difficulties (66.7%), cough (100%), and sore throat (33.3%). By contrast, average ages of influenza A/H1N1 (3.2 years) and B (23 years) positive groups among outpatients were lower than those among hospitalized patients (Tables 1 and 2). In addition, signs and symptoms were infrequently observed in outpatients. The results indicated no significant differences in clinical features between HCoV-NL63 and influenza infections, but co-infection of HCoV-NL63 and influenza A/H1N1 could cause more severe symptoms than single viral infection.

Phylogenetic tree analysis of HCoV-NL63 Taiwan isolates

For confirming HCoV-NL63 infection, the nucleotide sequences of 1a genes from Taiwan isolates were amplified by RT-PCR, sequenced, and then used to analyze a phylogenetic relationship with worldwide strains (Figure 3). Maximum likelihood (ML) analysis constructed a phylogenetic tree based on 1a gene nucleotide sequences of 39 global strains as references and 4 Taiwan isolates identified in the study. ML analysis of HCoV-NL63 viruses distinguished two clusters, and indicated Taiwan isolates exhibiting a genetically similarity with Cluster I, and forming a monophyletic clade with statistical significance (bootstrap >60%).

Discussion

This study showed prevalence of HCoV-NL63 infection in Taiwan during 2010-2011 post the 2009 H1N1 influenza virus pandemic outbreak. Positive rates of real time PCR for HCoV-NL63 detection were 8.4 % in hospitalized patients with pneumonia, but not found in outpatients with influenza-like illness (Tables 1 and 2). Phylogenetic analysis of Taiwan and global strains based on 1a gene revealed two clusters of HCoV-NL63 viruses (Figure 3), as similar patterns in previous reports.[5, 8] There was no difference in age-specific distribution between of HCoV-NL63 and influenza AH1N1 infections in hospitalized patients (Figure 1). However, the age distribution patterns of HCoV-NL63 infection in hospitalized patients differed from those of influenza A/H3N2 and B infections in hospitalized patients and outpatients (Tables 1 and 2). The results differed from the previous reports that low prevalence of HCoV-NL63 infection was detected in children, such as 1.2% in Japan,[7] 1.3% in Taiwan,[8] 2.1% in Australia,[4] 2.3% in Belgium,[9] 2.5% in Canada,[10] and 7% in Swiss.[11] Recently, the positive rate of HCoV NL63 in healthy control group (8.5%) was higher than those with upper respiratory tract infection (5.1%) in Ghana.[16] The study was the first report with the high risk of HCoV-NL63 infection for hospitalized patients with pneumonia. This study also identified 3 inpatients aged > 40 years co-infected with HCoV-NL63 and influenza A virus. Co-infection caused significantly higher rates of

253 breathing difficulties, cough and sore throat than those of single infection with
254 HCoV-NL63 and influenza A/H1N1. Therefore, HCoV-NL63 infection could be a
255 considerable impact on public health.

256 The study showed the seasonality of HCoV-NL63 infection as late winter,
257 overlapping the second peak of influenza epidemic in Taiwan (Figure 2). The
258 seasonality of HCoV-NL63 infection in Taiwan during 2010-2011 was similar to
259 those in temperate countries,[6, 7, 9, 10, 12] but different from some reports in which it was
260 in autumn in Taiwan during 2004-2005,[8] summer and autumn in Chongqing,[17] and
261 summer in Hong Kong.[5] Clinical manifestation analysis indicated fever (88.88%),
262 cough (77.78%), and pneumonia (100%), but no significant association with the group
263 of HCoV-NL63 infection compared to influenza A positive groups among hospitalized
264 patients (Table 1). In France, a survey of patients <20 years indicated more than one
265 third of the patients infected by HCoV-NL63 as bronchiolitis and pneumonia.[12] In
266 Brazil, a 46-year-old female patient with HCoV-NL63 infection had haemorrhagic
267 pneumonia, respiratory and renal failure, and died.[18] Thus, our results demonstrated
268 HCoV-NL63 infection correlating with severe lower respiratory tract diseases in
269 adults, implying HCoV-NL63 infection as a higher risk of severe respiratory illness
270 for adults than children.

271 In sum, real time PCR assay identified the overall positive rate of HCoV-NL63

infection as 8.4% in hospitalized patients with pneumonia in Taiwan during 2010-2011. Proportion of HCoV-NL63 infection in each age-specific group indicated HCoV-NL63 as high risk for pneumonia patients aged 16-25 and 26-35. Prevalence of HCoV-NL63 was predominant in late winter; co-infection with HCoV-NL63 and influenza A/H1N1 was associated with pneumonia in older adults. Phylogenetic analysis indicated HCoV-NL63 strains in Taiwan as in one of two major clusters based on 1a gene sequences of global strains, showing a very low level of heterogeneity between Taiwan and global strains. Transmission of HCoV-NL63 in older adults causes severe low respiratory tract diseasesHCoV-NL63. Results afford better understanding of epidemiology of HCoV-NL63 in Taiwan and contribute information necessary for worldwide surveillance of HCoV-NL63 infection.

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Potential conflicts of interest.

All authors report no conflicts of interest relevant to this article.

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Figure captions

Figure 1. Age distribution HCoV-NL63 (A-B) and influenza virus (C-D) infection in hospitalized patients with pneumonia (A, C) and outpatients with influenza-like illness (B, D).

Figure 2. Seasonal prevalence of HCoV-NL63, influenza A/H1N1, A/H3N2 and B viruses during 2010-2011 in Taiwan. Positive numbers (Left) and percentage (Right) of HCoV-NL63 infection in hospitalized patients (A), as well as influenza A/B infection in hospitalized patients (B) and outpatients (C).

Figure 3. Phylogenetic tree of partial 1a gene sequences from Taiwan and global HCoV-NL63 strains using Maximum-likehood method. Phylogenetic tree plots nucleotide sequences of HCoV-NL63 strains in this study and worldwide strains. Sequences were aligned via BioEdit and Clustal_X, phylograms generated by ML methods and MEGA tree-drawing software. Branch labels represent stability of branches over 1,000 bootstrap replicates, only bootstrap values >60% presented.

Table 1. Clinical data of hospitalized patients with pneumonia in this study.

	Hospitalized patients with pneumonia					
	Negative*	HCoV NL63 positive	Flu A/H1N1 positive	Flu A/H3N2 positive	Flu B positive	HCoV NL63 positive/ Flu A/H1N1 positive[+]
Cases	70	9	17	9	5	3
Average age (Y)	37.3	41.7	42.4	56.1	45.6	58.7
Clinical symptom (%)						
Breathing difficulties	48.6%	55.6%	52.9%	33.3%	60.0%	66.7% [a]
Cough	60.0%	77.8%	82.4%	88.9%	100.0%	100% [b]
Fever	88.6%	88.9%	82.4%	77.8%	80.0%	66.7%
Pneumonia (chest X-ray)	100.0%	100.00%	100.0%	100.0%	100.0%	100%
Myalgia	20.0%	44.4%	41.2%	11.1%	20.0%	33.3%
Sore throat	2.9%	11.1%	11.8%	22.2%	20.0%	33.3%[b]
Vomiting	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Muscle spasm	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Herpes angina	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Headache	4.3%	0.0%	0.0%	0.0%	0.0%	0.0%
Encephalitis	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%

*Negative for real-time PCR detection of HCoV NL63, influenza A/H1N1, influenza A/H3N2, and influenza B viruses.
[+] Patients co-infected with HCoV NL63 and influenza A/H1N1. [a]*P* value = 0.037 (Negative or Flu A/H1N1 positive vs co-infection); [b]*P* value <0.02 (Negative, HCoV NL63 positive or Flu A/H1N1 positive vs co-infection)

Table 2. Clinical data of the outpatients with influenza-like illness in this study.

	Outpatients with influenza-like illness					
	Negative*	HCoV NL63 positive	Flu A/H1N1 positive	Flu A/H3N2 positive	Flu B positive	HCoV NL63 positive/ Flu A/H1N1 positive[+]
Cases	146	0	23	5	1	0
Average age (Y)	9.0		3.2	52.0	23.0	
Clinical symptom (%)						
Breathing difficulties	5.5%		0.0%	20.0%[a]	0.0%	
Cough	14.4%		4.3%	20.0%	0.0%	
Fever	30.8%		8.7%	80.0%[a]	0.0%	
Pneumonia (chest X-ray)	1.4%		4.3%	40.0%[a]	0.0%	
Myalgia	2.7%		0.0%	40.0%[a]	0.0%	
Sore throat	0.7%		0.0%	20.0%[a]	0.0%	
Vomiting	3.4%		0.0%	0.0%	0.0%	
Muscle spasm	0.7%		0.0%	0.0%	0.0%	
Herpes angina	10.3%		0.0%	0.0%	0.0%	
Headache	0.0%		0.0%	0.0%	0.0%	
Encephalitis	0.7%		0.0%	0.0%	0.0%	

*Negative for real-time PCR detection of HCoV NL63, influenza A/H1N1, influenza A/H3N2, and influenza B viruses

[+] Two patients co-infected with HCoV NL63 and influenza A/H1N1 and one co-infected with HCoV NL63 and influenza A/H3N2

[a] *P* value <0.02 (negative or Flu A/H1N1 positive vs Flu A/H3N2 positive)

Fig. 1

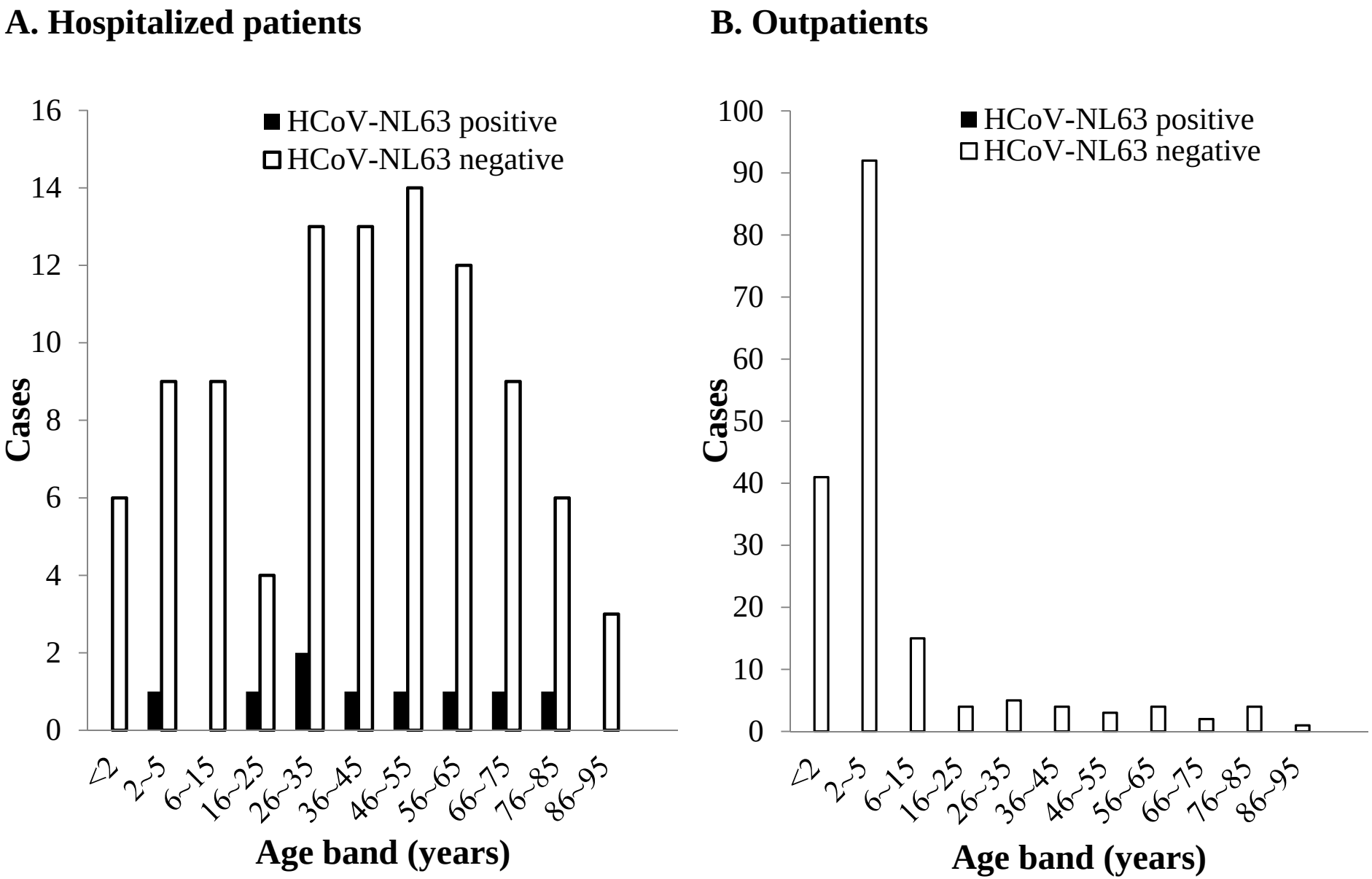

Fig. 1

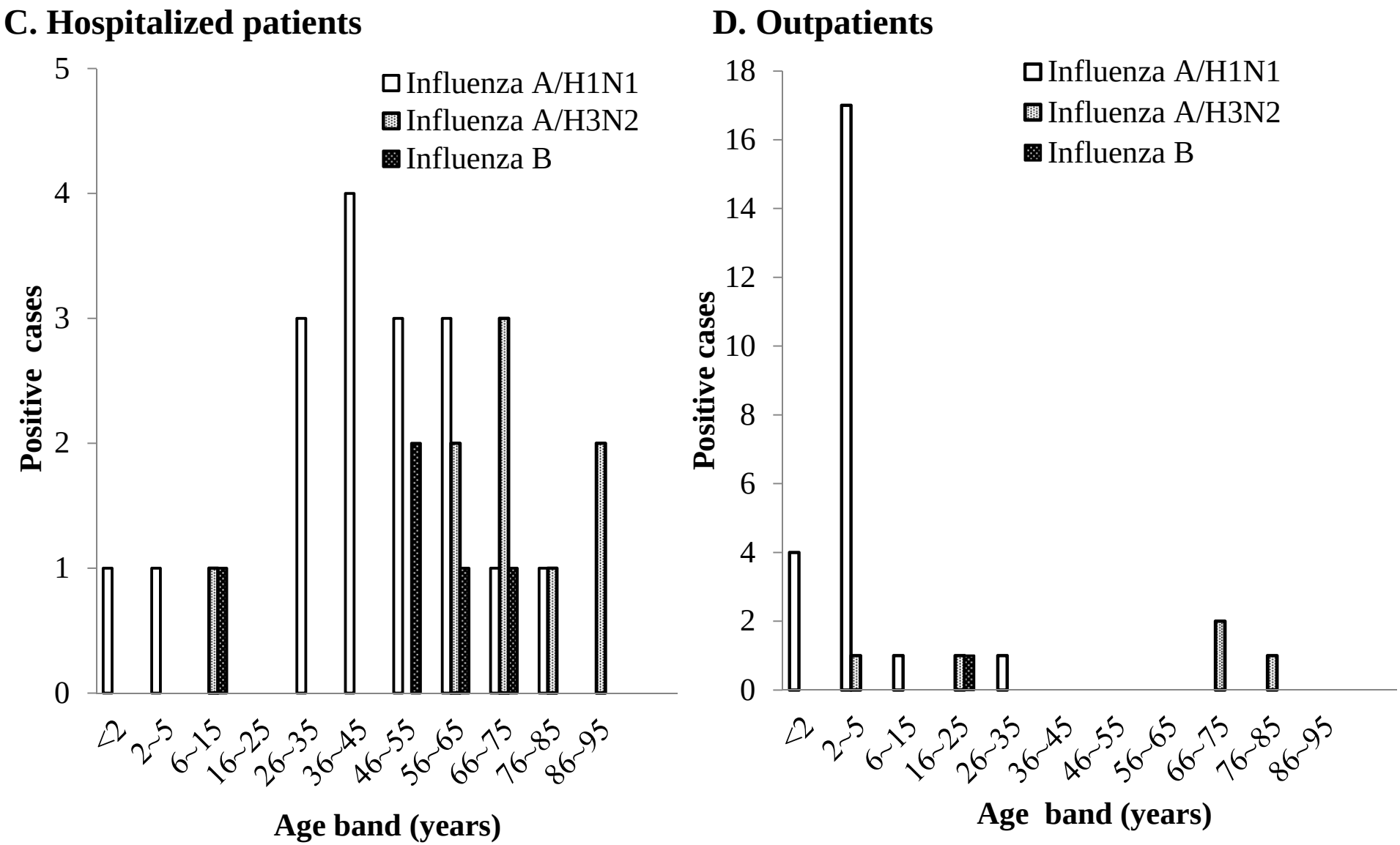

Fig. 2

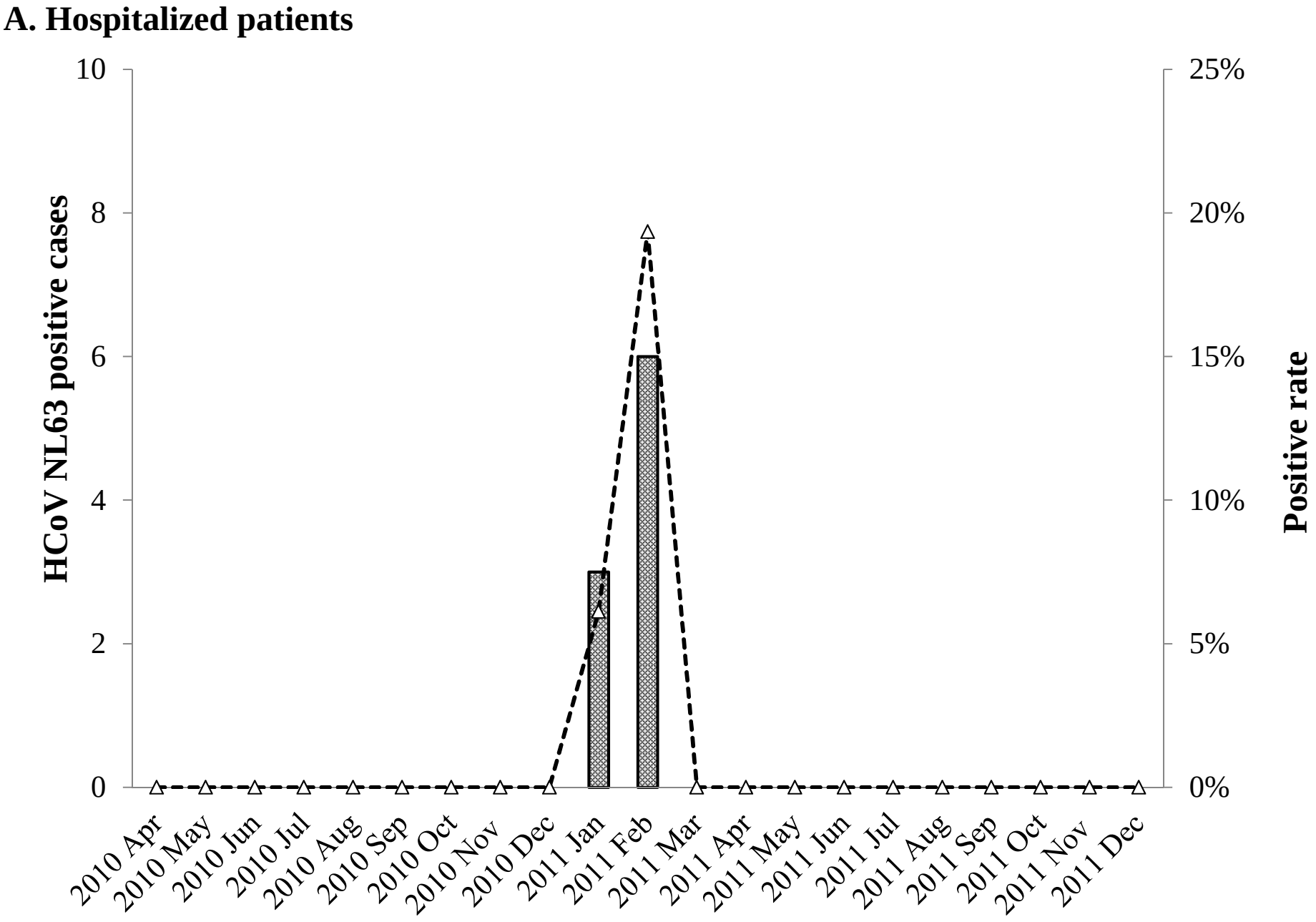

Fig. 2

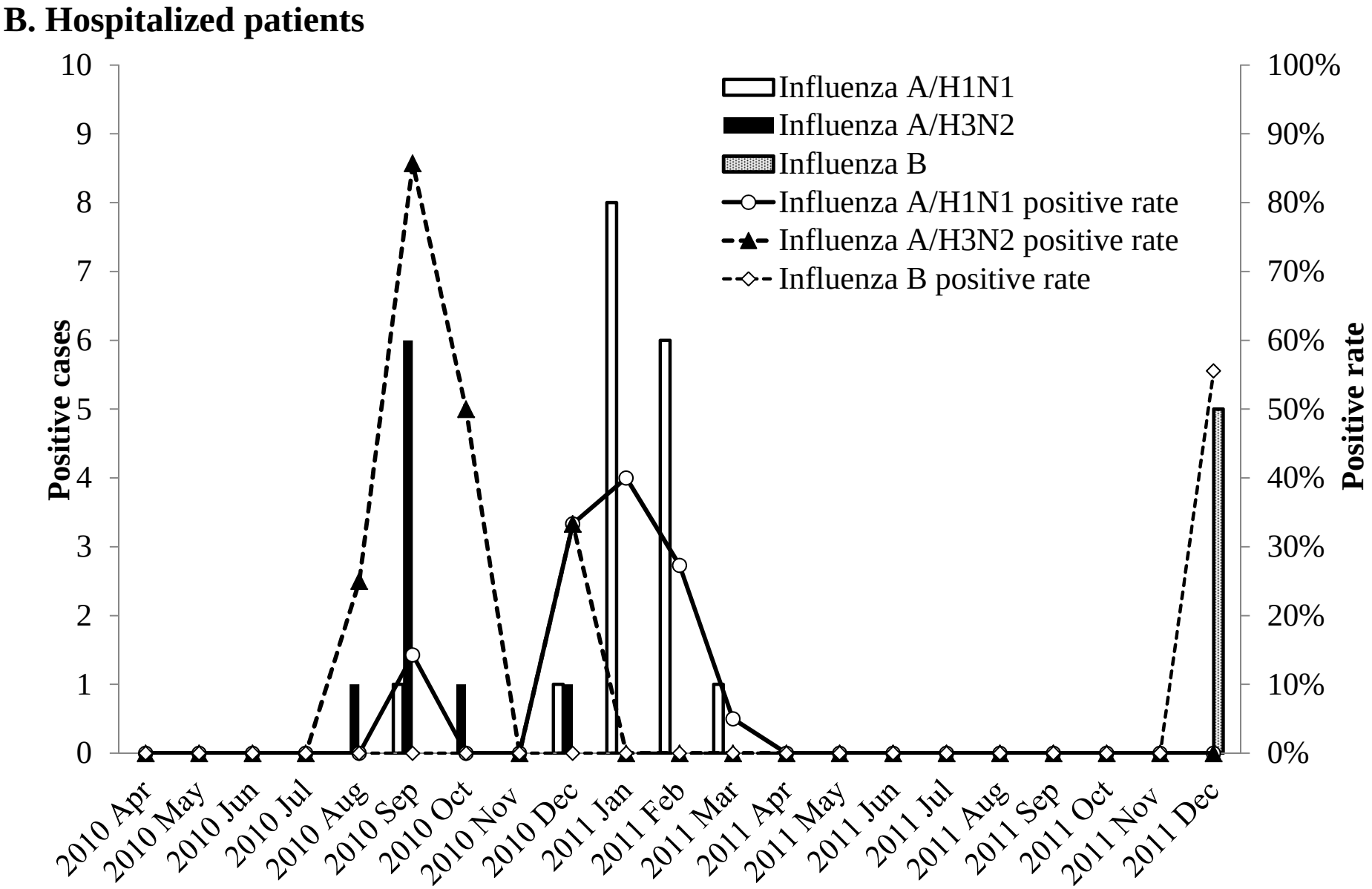

Fig. 2

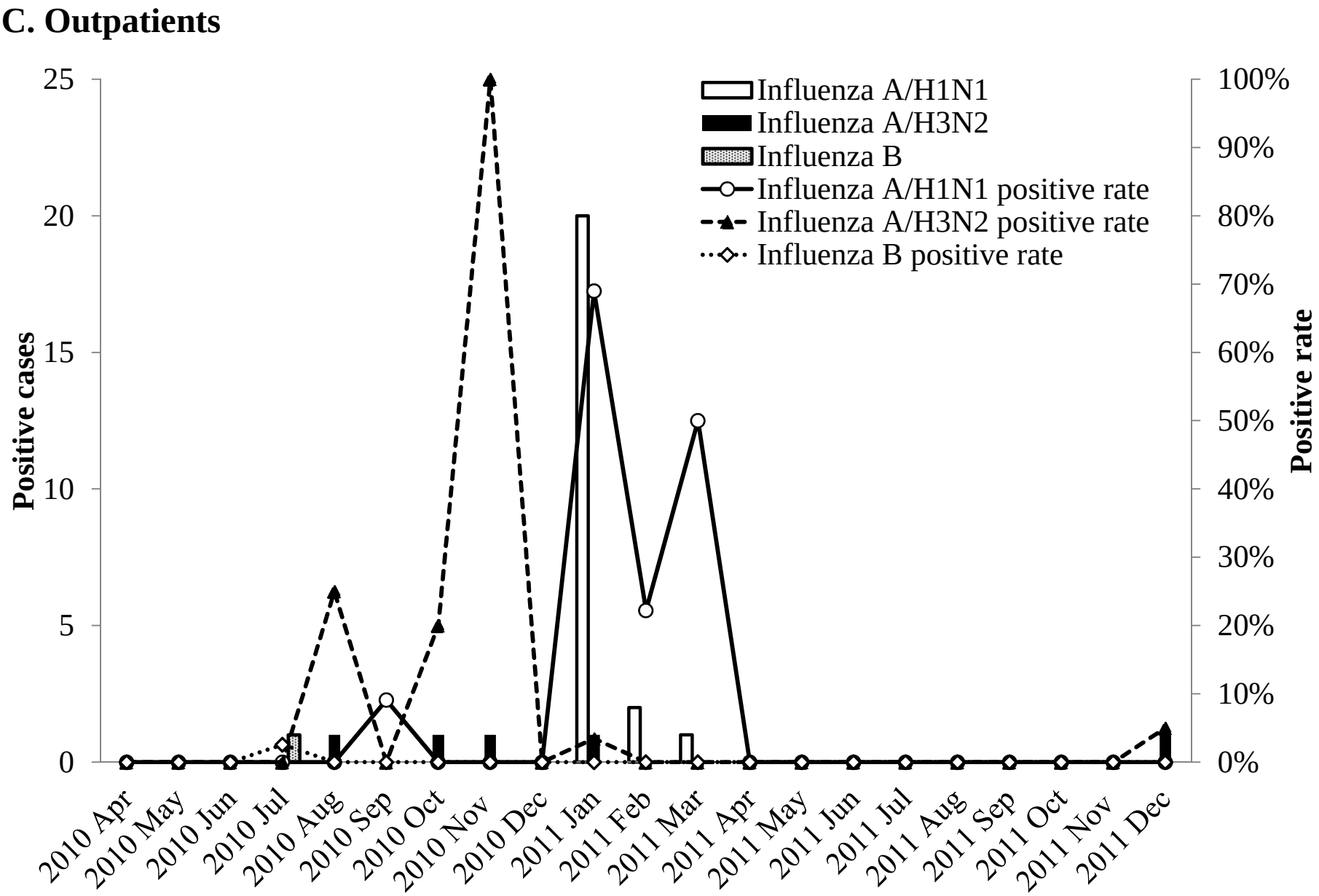

Fig. 3